# New dimeric inhibitor of heterologous α-amylases encoded by a duplicated gene in the short arm of chromosome 3B of wheat (*Triticum aestivum* L.)

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A new wheat dimeric α-amylase inhibitor, designated WDAI-3, has been characterized. WDAI-3 is a homodimeric protein active against α-amylase from human saliva and from the insect *Tenebrio molitor*, but inactive against that from pig pancreas or against trypsin. Its N-terminal amino acid sequence is closer to those of the wheat dimeric inhibitors 0.19 and 0.53 (89–91% identical positions in 44 residues) than to that of the monomeric 0.28 inhibitor (69% identical positions). *Iha-B1-2*, the gene encoding the new inhibitor, is located in the short arm of chromosome 3B, where it is part of an intrachromosomal gene duplication that also codes for the 0.53 inhibitor.

Up to ten families of proteinaceous inhibitors of heterologous hydrolytic enzymes (proteases and α-amylases) have been included in a recent review on the subject [1]. These inhibitors are currently attracting considerable attention because of their possible implication in defense mechanisms [1–4]. Studies concerning their chemistry and genetic control, as well as their manipulation in plant breeding, are therefore relevant in this context.

One of the protein families, which includes both trypsin inhibitors and the subunits of monomeric, dimeric and tetrameric inhibitors of heterologous α-amylases, has been extensively studied in several cereal species, especially in wheat and barley [1, 5, 6], where they represent a substantial fraction of the grain protein. This group of inhibitors is encoded by a multigene family which is dispersed over several chromosomes [1, 7–14], and is homologous to the sulphur-rich domain of major cereal prolamins [15]. In wheat, some of the monomeric and dimeric α-amylase inhibitors have been characterized [1, 5, 16] and, in particular, the complete amino acid sequences of two dimeric (0.53 and 0.19) and one monomeric (0.28) inhibitor have been determined [17–19]. Genes for inhibitors 0.53 and 0.19 have been assigned to the short arms of chromosomes 3B and 3D, respectively, whereas the location of the gene for inhibitor 0.28 has been found in the short arm of chromosome 6D [11].

Further knowledge of the inhibitory properties of different components of this protein family and of the chromosomal distribution of their corresponding genes is warranted in connection with an assessment of their possible plant protection role. We report here a new wheat dimeric inhibitor of heterologous α-amylases and the chromosomal location of the corresponding gene, which, together with the gene encoding inhibitor 0.53, represents a duplication in the short arm of chromosome 3B of hexaploid wheat (*Triticum aestivum* L.; genomes AABBDD).

## MATERIALS AND METHODS

### *Isolation and characterization of the inhibitor*

A crude inhibitor preparation was obtained from *Triticum turgidum* (genomes AABB) cv. Senatore Capelli by 0.15 M NaCl extraction and $(NH_4)_2SO_4$ precipitation as previously described [12, 20]. This preparation was subjected to gel filtration on Sephadex G-100 and fractions around 25 kDa which included the dimeric inhibitors, were pooled. The pooled fractions were chromatographed on a preparative high-performance liquid-chromatography (HPLC), reverse-phase column (Vydac-$C_4$, 22 mm × 250 mm, particle size 10 μm), using a two-step linear gradient 20–50% acetonitrile in 0.1% trifluoroacetic acid (2 ml/min; linear 20–35% gradient in 140 min, linear 35–50% gradient in 100 min).

HPLC gel filtration was carried out on a Spherogel TSK-G300 column (21.5 mm × 300 mm). Purified proteins (50 μg) were eluted at 1 ml/min with 100 mM ammonium acetate, pH 6.8, with or without 0.1% SDS, as indicated in the text. A monomeric (0.28) and a dimeric (0.19) inhibitor, purified as previously described [11], were used for comparison.

Protein sequencing was performed by standard methods, using an Applied Biosystems 470A gas-phase sequenator.

Inhibition of different α-amylases was tested essentially according to Benfeld [21], with a 20 mM NaCl, 0.1 mM $CaCl_2$, pH 5.4 buffer for *Tenebrio molitor* α-amylase, a 20 mM potassium phosphate, 67 mM NaCl, 0.1 mM $CaCl_2$, pH 6.9, buffer for human saliva α-amylase, and the same buffer at pH 7.6 for pig pancreas α-amylase. 1 unit enzyme was used/assay, defined as the amount of enzyme required to produce the reducing equivalents of 1 μmol maltose in our experimental conditions. Trypsin inhibition was tested as in Boisen and Djurtoft [22]. Protein concentration was determined by standard methods [23, 24]. Polyacrylamide gel electrophoresis in sodium dodecyl sulphate, SDS/PAGE [25], and two-dimensional electrophoresis by combined electrofocusing and starch-gel electrophoresis were carried out as previously described [26].

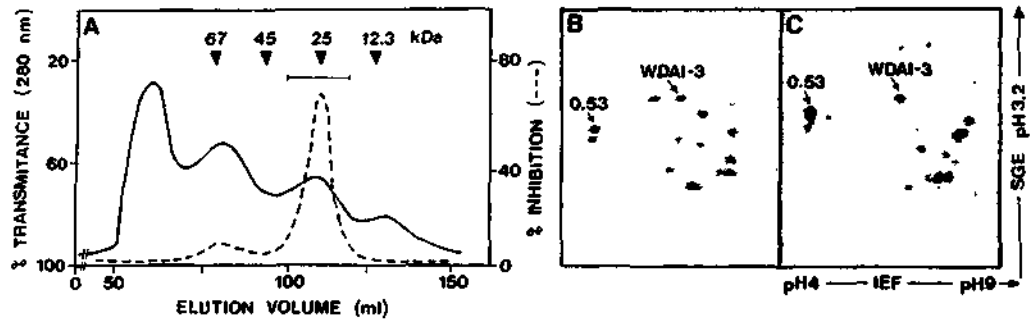


Fig. 1. *(A) Gel filtration on Sephadex G-100 of a 150 mM NaCl extract precipitated with 50% saturated $(NH_4)_2SO_4$ (crude inhibitor preparation) from tetraploid wheat.* The fraction indicated by a horizontal bar was used for further purification steps. Bovine serum albumin (67 kDa), ovalbumin (45 kDa), chymotrypsinogen (25 kDa) and cytochrome *c* (12.3 kDa) were the standards used to calibrate the column. Inhibitory activity against salivary α-amylase was assayed in appropriate aliquots from every second tube. *(B, C) Two-dimensional electrophoretic maps (electrofocusing, IEF × starch-gel electrophoresis, SGE) of the crude inhibitor preparation (B) and of the 25-kDa gel-filtration fraction (C) indicated in (A).* Arrows point to the positions of WDAI-3, and of the dimeric α-amylase inhibitor 0.53

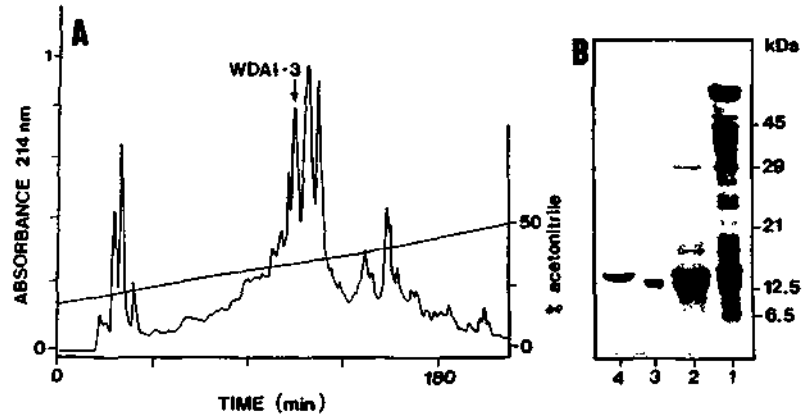


Fig. 2. *(A) HPLC fractionation on Vydac-$C_4$ column of the 25-kDa gel-filtration fraction shown in Fig. 1A.* The peak corresponding to WDAI-3 is indicated. *(B) SDS-PAGE of samples from tetraploid wheat endosperm.* (1) Crude inhibitor preparation; (2) 25-kDa gel-filtration fraction from Fig. 1A; (3) purified WDAI-3; (4) purified 0.53 α-amylase dimeric inhibitor. The molecular masses of reference proteins in kDa appear on the right side of the figure

*Genetic analysis*

Compensated nulli-tetrasomic and ditelosomic lines of *T. aestivum* cv. Chinese Spring (gift of E.R. Sears, Columbia, MO, USA) were used to locate the structural gene for the inhibitor. The 70% ethanol extracts of these genetic stocks were fractionated by two-dimensional electrophoresis as described above. The precise location of spots corresponding to purified proteins was determined by co-electrophoresis with the euploid extract and with that of the appropriate aneuploid line.

## RESULTS

*Isolation and characterization of inhibitor WDAI-3*

A crude inhibitor preparation was obtained from tetraploid wheat endosperm and then fractionated by gel filtration on Sephadex G-100. The fraction with apparent molecular mass around 25 kDa, which included the dimeric inhibitors and was highly active against human salivary α-amylase (Fig. 1A), was shown to consist of two main components and several minor ones by two-dimensional electrophoresis (Fig. 1B, C). One of the two main components was identified as the previously characterized dimeric inhibitor 0.53 [11, 19] by co-electrophoresis with an authentic sample (not shown). The second main component was obtained by preparative HPLC of the 25-kDa gel filtration fraction (Fig. 2A). Homogeneity of this component (WDAI-3) was checked by two-dimensional electrophoresis (not shown) and by SDS/PAGE, where it appeared with a molecular mass of 12.7 kDa (Fig. 2B). WDAI-3 was shown to form dimers by gel filtration on a Spherogel TSK-G300 HPLC column. Under non-dissociating conditions, it eluted as the previously reported dimeric inhibitor 0.19 and at a lower elution volume than the monomeric inhibitor 0.28, whereas under dissociating conditions, the same elution volume was obtained for the three inhibitors (Fig. 3).

The first 44 residues of its N-terminal amino acid sequence were determined and aligned with those of previously known monomeric and dimeric inhibitors (Fig. 4). Although a high similarity of WDAI-3 to both types of inhibitors was evident, the sequence of the new inhibitor was closer to those of the dimeric ones (Fig. 4).

The new inhibitor was about tenfold more active than the monomeric 0.28 inhibitor against human salivary α-amylase (Fig. 5A), and about one-tenth as active as 0.28 against insect α-amylase (Fig. 5B). None of the tested inhibitors was active against the α-amylase of pig pancreas (up to 2 μg inhibitor/assay) or against trypsin (up to 10 μg/assay).

*Chromosomal location of the gene encoding WDAI-3*

The chromosomal location of the gene encoding WDAI-3, designated *Iha-B1-2*, was investigated by analysis of the compensated nulli-tetrasomic and the ditelosomic series of lines of hexaploid wheat cv. Chinese Spring. Two-dimensional electrophoresis of the 70% ethanol extracts was chosen as the method of analysis because it had been previously shown that the inhibitors were readily extractable with this solvent, and that the corresponding two-dimensional patterns were simpler and with less overlapping than those obtained for the $H_2O$ or 0.15 M NaCl extracts [7, 9, 11]. The presence of WDAI-3 in hexaploid wheat was demonstrated by co-electrophoresis of the purified inhibitor with the hexaploid wheat extract (not shown). The spot corresponding to WDAI-3 was present in all lines tested except those lacking either chromosome 3B or just its short arm (Fig. 6). The spot corresponding to inhibitor 0.53 was also missing in the same genetic stocks, in agreement with a previous report [11].

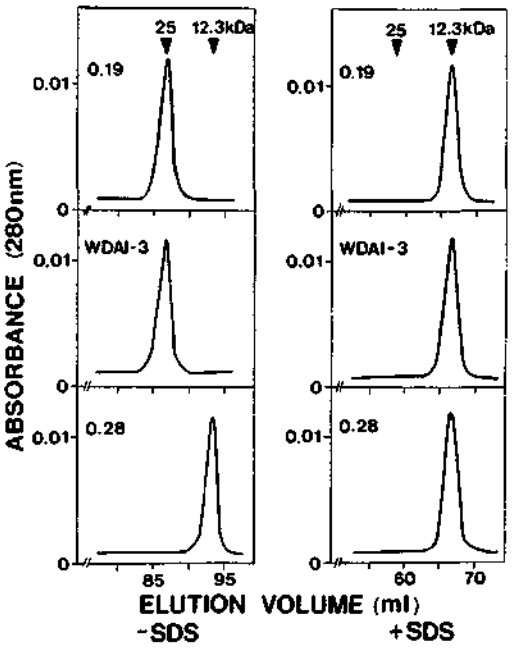


Fig. 3. *HPLC gel filtration on a Spherogel TSK-G300 SWG column of the indicated purified inhibitors.* The elution buffer was 100 mM ammonium acetate, pH 6.8, or the same buffer with 0.1% SDS. Arrow heads point to the elution volumes of chymotrypsinogen (25 kDa) and cytochrome *c* (12.3 kDA)

## DISCUSSION

The new inhibitor, WDAI-3, is one of the major components of the crude inhibitor preparation (Fig. 1) and of the low-molecular-mass fraction from the 70% ethanol extract of wheat (Fig. 6). WDAI-3 is extracted as a dimer (Fig. 1A) and is able to self-associate *in vitro* (Fig. 3). Its dimeric nature is further corroborated by its amino acid sequence, which is closer to previously described wheat dimeric inhibitors than to the monomeric one [17–19]. The fact that, as is the case for the other dimeric inhibitors [5, 16], WDAI-3 is significantly more active than the monomeric 0.28 inhibitor against salivary α-amylase, and markedly less active than 0.28 towards the enzyme from the insect *T. molitor*, suggests that there are not significant differences in specificity within the dimeric class of inhibitors, which are more active against salivary α-amylase than against the insect α-amylase, whereas the opposite is true both for the monomeric and the tetrameric inhibitors [1, 5, 12, 27]. It will be of interest if other possible minor components of the dimeric class have the same or different specificity as those already reported.

The location of gene *Iha-B1-2* on the same chromosome arm (3BS) as the gene encoding the 0.53 dimeric inhibitor [11], which is clearly homologous to WDAI-3 (91% identical

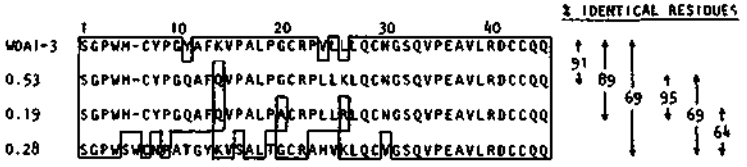


Fig. 4. *Aligment of the N-terminal sequence of WDAI-3 with those of the wheat dimeric inhibitors 0.19 and 0.53 [18, 19] and the wheat monomeric inhibitor 0.28 [17].* Percentages of identical residues for all binary comparisons are listed at the right of the figure

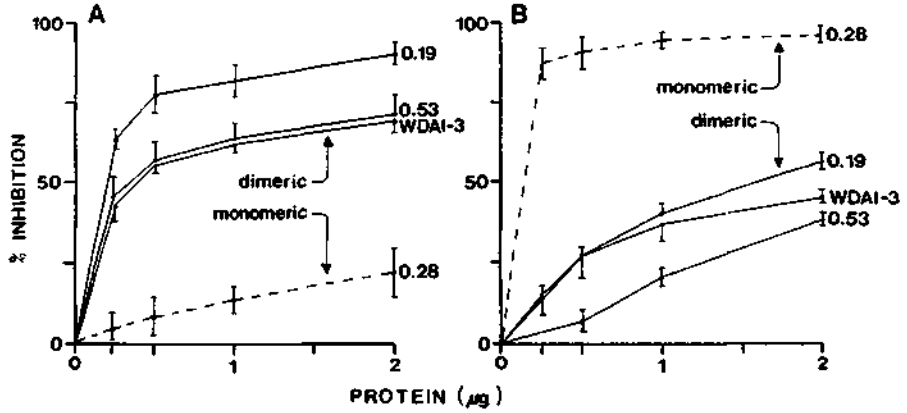


Fig. 5. *Inhibitory activities against the α-amylases from human saliva (A) and from the insect* Tenebrio molitor *(B) of the indicated wheat inhibitors.* Inhibition tests were carried out using 1 unit α-amylase (see Materials and Methods). Vertical bars indicate standard deviations


Fig. 6. *Two-dimensional electrophoretic protein maps (electrofocusing, IEF, and starch-gel electrophoresis, SGE) of 70% ethanol extracts from individual endosperms.* (A) *T. turgidum* cv. Senatore Capelli. (B) *T. eastivum* cv. Chinese Spring. (C) Ditelosomic 3BL. (D) Ditelosomic 3BL plus 10 μg purified WDAI-3. Arrows point to the positions of WDAI-3, inhibitor 0.19 and inhibitor 0.53

residues in 44 positions), indicates that an intrachromosomal gene duplication has occurred in chromosome 3B. On the basis of the N-terminal 44 amino acid residues, dimeric inhibitor 0.19, whose gene is on the short arm of chromosome 3D [11], seems to be closer to inhibitor 0.53 than WDAI-3 (95% vs. 91% identical positions). This suggests that, excluding some special type of sequence homogeneization, the duplication must have occurred before the evolutionary branching out of *Triticum tauschii*, donor of the D genome to cultivated wheat, and the unknown donor of the B genome.

The considerable divergence that has occurred within this protein family, which is reflected in the diversification of amino acid sequences and of other structural features, in the dispersion of the corresponding genes by duplication and translocation [1, 5, 13], and in the range of inhibitory specifities that it represents, is consistent with a protective role against predators. However, the evidence presented here confirms previous indications [1, 5, 8, 28] of the low intraspecific variability of individual members of the family (i.e. WDAI-3 has been conserved across the hexaploidization step) and of the not-recent origin of the observed divergence (i.e. ancient origin of the duplication). This suggests that the putative defense system would be in place in order to meet long-standing challenges rather than rapidly evolving in response to emerging ones.

No other function has been postulated for this protein family, except for a possible reserve role [1]. Presumably, this less specific role should have allowed a greater intraspecific variability than that observed at the different loci [1].

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